PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12P 21/02, C12N 5/00
C07K 15/00

A1

(11) International Publication Number: WO 94/11525

(43) International Publication Date: 26 May 1994 (26.05.94)

(21) International Application Number: PCT/DK93/00375

(22) International Filing Date: 17 November 1993 (17.11.93)

(30) Priority data:
07/977,356 17 November 1992 (17.11.92) US
08/044,865 8 April 1993 (08.04.93) US

(71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors: RASMUSSEN, Mirella, Ezban ; Abildgaardsgade 24, DK-2100 Copenhagen (DK). KONGERSLEV, Leif ; Teglvaerksvej 51, DK-3460 Birkeroed (DK).

(81) Designated States: AU, BG, BR, BY, CA, CZ, FI, HU, JP, KP, KR, NO, NZ, PL, RO, RU, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: IMPROVED RECOMBINANT PRODUCTION OF PROTEINS HAVING FACTOR VIII:C ACTIVITY

(57) Abstract

A method for effecting an increased expression of recombinant proteins, especially proteins having Factor VIII:C activity in the presence of heparin is disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Improved recombinant production of proteins having factor VIII:C activity.

TECHNICAL FIELD

This invention relates to a method for effecting an increased expression of recombinant proteins, especially proteins having Factor VIII:C activity.

BACKGROUND OF THE INVENTION

Hemophilia A is an X-chromosome-linked inherited disease which afflicts 1-2 males per 10,000. The desease is caused by an absence of deficiency of Factor VIII:C. Factor VIII:C is a very large glycoprotein (native M_r 330 K - 360 K), which is present in plasma at extremely low concentrations. It is a necessary element in the proteolytic cascade which converts soluble fibrinogen to insoluble fibrin, forming a clot to prevent blood loss from traumatized tissue. In the bloodstream, it is found in noncovalent association with von Willebrand factor (vWF), which acts as a stabilizing carrier protein. Factor VIII:C is very susceptible to cleavage by thrombin, plasmin, activated protein C, and other serine proteases. It is generally isolated from plasma or plasma products as a series of related polypeptides ranging from M_r 160 K-40 K with predominant species of M_r 92 K and M_r 80 K-77 K. This complex pattern has made the analysis of the structure of active Factor VIII:C very difficult.

Factor VIII:C and the related polypeptides have been described by F. Rotblat et al, Biochemistry (1985) 24:4294-4300; G.A. Vehar et al, Nature (1984) 312:337-342; J.J. Toole et al, Nature (1984) 312:342-347; and M.A. Truett et al, DNA (1985) 4:333-349. E. Orr et al, Molecular Genetics of Clotting Factors, p. 54, s321. The sequence has been reported by J.J. Toole et al, supra; W.I. Wood et al, Nature (1984) 312:330-336;

and M.A. Truett et al, supra. The full-length protein contains three repeats of one sequence (I), and two repeats of a second sequence (III). A third, heavily glycosylated sequence (II) is present between the second and third I repeats, and is apparently cleaved proteolytically to form the M_r 92 K and M_r 80 K polypeptides. The first two I repeats form the A domain, while the third I repeat and the two III repeats form the C domain. The II sequence forms the B domain. Thus, the full-length protein has the structure I_1-I_2-II-I_3-III_1-III_2 (A-B-C), while the M_r 92 K and M_r 80 K polypeptides (A and C) have the structures I_1-I_2 and I_3-III_1-III_2, respectively. R.L. Burke et al, J Biol Chem (1986), have shown by expression of the 92 K and 80 K polypeptides that both peptides are necessary for Factor VIII:C activity.

Factor VIII:C has historically been isolated from blood in a concentrated form for therapeutic treatment of hemophilia. However, concerns regarding transmission of HIV and other blood-borne diseases have stimulated activity to provide alternative supplies of Factor VIII:C. It is of substantial interest to be able to supply compositions having Factor VIII:C activity without concerns as to the transmission viral diseases associated with the native Factor VIII:C.

The recombinant proteins having Factor VIII:C activity which are prepared according to the present invention may be full length Factor VIII:C corresponding to the protein isolated from plasma, or a derivative thereof having the capability of normalizing the insufficient blood clotting caused by deficiency of Factor VIII:C. The derivatives of Factor VIII:C may be shortened single chain forms or derivatives comprising two chains. Even fragments of Factor VIII:C which may not per se show coagulant activity, but which may be used in the treatment of haemophiliacs e.g. for saturation of antibodies against Factor VIII:C present in inhibitor patients.

The proteins produced in accordance with the present invention

show homology with all or a part of the natural Factor VIII:C molecule.

The preparation of recombinant proteins having Factor VIII:C activity by recombinant techniques has inter alia been disclosed in a number of publications. Thus, European Patent Application No. 160 457 and International Patent Application No. WO 86/01961 disclose the production of full length Factor VIII:C, and European Patent Application No. EP 150 735, International Patent Application No. WO 86/06101, European Patent Application No. EP 232 112, International Patent Application No. WO 87/04187, International Patent Application No. WO 87/07144, International Patent Application No. WO 88/00381, European Patent Application No. EP 251 843, European Patent Application No. EP 253 455, U.S. Patent No. 4.980.456, European Patent Application No. EP 294 910, European Patent Application No. EP 265 778, European Patent Application No. EP 303 540, International Patent Application No. WO 91/07490, and International Patent Application No. WO 91/09122 disclose recombinant expression of shortened single chain forms or subunits of Factor VIII:C or co-expression of subunits for the production of complexes showing coagulant activity or binding affinity to antibodies inhibiting Factor VIII:C.

Expression of recombinant full-length human Factor VIII:C is usually low and the molecure is unstable due to proteolysis.

The derivatives of Factor VIII:C in the form of shortened single chain forms or derivatives comprising two chains have also been successfully produced by recombinant techniques. Although these derivatives normally are expressed in a higher yield than full-length Factor VIII:C, there is still a desire for increasing the level of xpression.

It is d scribed in WO 87/04187 and EP 251 843 that expression of Factor VIII:C in the presence of von Willebrand Factor (vWF) or phospholipide increases the expression of Factor VIII:C. In

EP 441 695 it is disclosed to express Factor VIII:C or an analogue thereof in the presence of a cationic or anionic polymer, preferably a polysaccharide which most preferred is in a sulphatizes form. Among other additives heparin was tested in an amount of 10 to 80 IU/ml. The addition of heparin only "showed a very limited to no effect" on the expression of Factor VIII delta 2 having a deletion of amino acids 771 to 1666, as disclosed in EP 303 540 as compared to serum fre medium. The expression level is slightly increased with increased concentration of heparin. However, the expression level is far below the expression level in the presence of serum or vWF.

DISCLOSURE OF THE INVENTION

The present invention relates to a method for effecting an increased expression of recombinant proteins having Factor VIII:C activity in a host cell being able to express said protein comprising culturing said host i a cell growth medium comprising heparin in a very low concentration below 10IU/ml so as to express said protein.

DETAILED DESCRIPTION OF THE INVENTION

It has surprisingly been found that the addition of heparin in small amounts below 10IU heparin/ml increases the expression of recombinant proteins showing Factor VIII:C activity and stabilizes the product to an extent leading to an increased yield of more than 50%. The stabilizing effect of heparin is indicat d through the observed reduction of the activation of the 92 kD subunit by prot ases. Such addition may, according to the invention, be made to cell growth medium comprising serum, preferably in the form of fetal or n w born calf serum, and to serum free medium comprising lipoprotein, vWF, or phospholipid or other additional constituents used for increasing the

expression in serum free media in order to obtain the improved expression.

The lipoprotein used in the method of the invention may e.g. be lipoproteins as described in EP 254076. Such lipoproteins are commercially available under the Trade Mark EX-CYTE. The lipoprotein may also be isolated from egg yolk, for example by the method described in Immunological Communications 9 (5), 475-493 (1980).

Phospholipids used in the method of the invention may e.g. be such phospholipids as described in WO87/04187.

Other additional constituents used for increasing the expression in serumfree media may e.g. be an egg yolk fraction being free of lipoprotein and lipids.

According to a preferred aspect of the invention, heparin is added to a concentration of from 0.5 to 8 IU/ml, a concentration of from 1 to 2 IU/ml being most preferred.

The addition of heparin to a cell growth medium comprising serum is a preferred aspect of the invention giving rise to a considerable increase of the Factor VIII:C level.

Still more preferred in the addition of heparin to a cell growth medium comprising serum and further added lipoprotein giving rise to an extremely high level of Factor VIII:C on expression in suspension.

According to another preferred aspect of the invention, serum free cell culture medium is used in order to avoid the addition of constituents which might give rise to transference of blood borne diseases, such medium being supplemented with lipoprotein, von Willebrand Factor, phospholipid, or a combination of two or more of these.

In accordance with a more preferred aspect of the invention lipoprotein and heparin are added to a serum free medium. In a preferred embodiment about 5% of lipoprotein fraction and about 2IU of heparin are added giving rise to a very high expression of Fator VIII:C in the term of expressed Factor VIII:C activity.

According to another preferred aspect of the invention, heparin is added to a growth medium for culturing a host cell for expressing a complex of the 92kD and 80/77kD subunits of Factor VIII:C. For such cultivation heparin does not only increase the level of expression of the individual subunits of Factor VIII:C, but also increases the degree of complex formation and stabilizes the produced complex and thus, the yield of product showing coagulant activity.

The term "A domain" refers to that portion of human Factor VIII:C which constitutes the M_r 92 K protein subunit or the Factor VIII:C heavy chain (FVIII-HC). The A domain contains from about 740 to about 760 amino acids, and is found at the N-terminus of the native human Factor VIII:C. Of particular interest is an N-terminal chain having the entire sequence to the thrombin cleavage site at Arg_{740}-Ser_{741}.

The term "B domain" refers to that portion of native human Factor VIII:C which is generally removed by intracellular cleavage, and which is heavily glycosylated in human plasma and when expressed in mammalian cells such as COS7, CHO and BHK cells. The B domain contains an N-terminal sequence, which allows cleavage of the A domain from the B domain by thrombin. The B domain also has a C-terminal processing site which allows cleavage of the C domain from the A-B precursor by an enzyme located in the Golgi apparatus of the mammalian cell.

The term "C domain" ref rs to that portion of native human Factor VIII:C which constitutes th C-terminus of the full length protein, and is cleaved intracellularly to form the

Factor VIII:C light chain (FVIII-LC). The light chain will have an amino acid sequence substantially the same as the amino acid sequence of the C-terminus of a Factor VIII:C polypeptide, usually at least about 80%, more usually at least about 90% of the Factor VIII:C M_r 80 K chain, particularly beginning with amino acid 1640, preferably at about amino acid 1649, ±10 amino acids, more particularly ±1 amino acid, and continuing to at least about amino acid 2300, usually 2310, ±10 amino acids, preferably 2325, ±5 amino acids, more preferably to the terminal amino acid (2332). Usually, the light chain will have at least about 85%, more usually at least 95%, of the III_1-III_2 domains, desirably the I3-III1-III2 domains.

The term "co-expressing" as used herein refers to simultaneous expression of an A domain polypeptide (92 K) and a C domain polypeptide (80 K) within the same host cell. The polynucleotide sequences encoding the A and C domains may be on the same or on different expression cassettes or plasmids. Co-expression of the A and C domains permits proper folding to occur, which in turn provides an A-C complex having activity and efficiency of secretion.

The term "production medium" as used herein refers to any medium suitable for culturing host cells, and includes media suitable for obtaining expression of recombinant products whether or not actual cell "growth" occurs. Production media generally include nutrients and a metabolizable energy source in an aqueous solution. If desired, production media may also include a compound which induces expression of the recombinant polypeptides of the invention. Selection of such an inducing compound depends upon the promoter selected to control expression. Other typical additives include selection compounds (i.e., drugs or other chemicals added to the media to insur that only transformed host cells survive in the medium) and serum, such as fetal bovine serum (FBS).

The term "serum-free medium" is a solution which has been sup-

plemented to such an extent that the necessary trace factors present in serum need not be added in the form of serum. The serum free medium may be a synthetical medium not comprising components isolated from animal tissues or body fluids. There are many suitable cell growth media available from commercial sources.

The term "IU" as used herein in connection with heparin is defined by standardization against the 4th International Standard for Heparin (code-labelled 82/502) prepared by the National Institute for Biology and Standard and Control. London UK.

The term "homology" as used herein means identity or substantial similarity between two polynucleotides or two polypeptides. Homology is determined on the basis of the nucleotide or amino acid sequence of the polynucleotide or polypeptide. In general terms, usually not more than 10, more usually not more than 5 number%, preferably not more than about 1 number% of the amino acids in the chains will differ from the amino acids naturally present in the Factor VIII:C A and C domains. Particularly, not more than about 5%, more usually not more than about 1% will be nonconservative substitutions. Conservative substitutions include:

Gly ↔ Ala;	Lys ↔ Arg;
Val ↔ Ile ↔ Leu;	Asn ↔ Gln; and
Asp ↔ Glu;	Phe ↔ Trp ↔ Tyr.

Nonconservative changes are generally substitutions of one of the above amino acids with an amino acid from a different group (e.g., substituting Asn for Glu), or substituting Cys, Met, His, or Pro for any of the above amino acids.

The specific activity of a protein complex prepared according to the invention may be d termined by means known in the art, as described below (e.g., by using the commercially available Coatest assay).

The structural genes typically include a leader sequence coding for the signal peptide which directs the polypeptide into the lumen of the endoplasmic reticulum for processing and maturation. Optionally included are additional sequences encoding propeptides which are processed post-translationally by endopeptidases, where the endopeptidases cleave a peptide bond, removing the propeptide to generate the mature polypeptide. The signal peptide may be the naturally occurring one, particularly for the N-terminal peptide, or may be any signal peptide which provides for the processing and maturation of the polypeptides.

Various mammalian host cells may be employed in which the regulatory sequences and replication system are functional. Such cells include COS7 cells, Chinese hamster ovary (CHO) cells, mouse kidney cells, hamster kidney cells, HeLa cells, HepG2 cells, or the like, e.g VERO cells, W-138 or MDCK cell lines.

The expressed product can be purified by affinity chromatography using antibodies, particularly monoclonal antibodies directed against the FVIII-LC or FVIII-HC, chromatography, e.g. HPLC, electrophoresis, or extraction.

The subject method provides for production of a complex of the active chains (92 K and 80 K) which has Factor VIII:C activity. Production is evidenced by conditioned media as described in the experimental section, which will have at least about 1, usually at least about 5 U/mL, more usually at least about 10 U/mL of Factor VIII:C activity in the Coatest assay.

The proteins having Factor VIII:C activity produced according to the invention are primarily intended for treatment of hemophiliacs and patients suffering from other conditions involving blood clotting disorders. The subject proteins may be administered in physiologically acceptabl carrier, such as water, saline, phosphate buffered saline, and citrate buffer d saline, at concentrations in the range of about 10-200 U/mL.

See U.S. Patent Nos. 3,631,018; 3,652,530, and 4,069,216 for methods of administration and amounts. Other conventional additives may also be included. They also have a variety of uses as immunogens for the production of antibodies, for isolation of von Willebrand factor by affinity chromatography and in diagnostic assays for Factor VIII:C.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is described with reference to the drawings on which

Fig. 1 shows a titration of the heparin effect on level of Factor VIII:C units in suspension culture, and

Fig. 2 shows a gel illustrating the stabilizing effect of heparin on Factor VIII-HC

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

EXPERIMENTAL PART

MATERIALS AND METHODS

Heparin from DAK, Denmark, was used for all experiments.

Lipoprotein was isolated from egg yolk in the form of a fraction being rich in lipoprotein. The fractionation was carried out as disclosed in d tail in Example 6.

PEG 6000 from Merck, Catalogue No. 807491 was used for the

fractionation of egg yolk.

PBS used for the fractionation of egg yolk was made by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 in deionized water, adjusting the pH to 7.3 by adding 1M HCl/NaOH and adding deionized water ad 1 liter. All chemicals were from Merck.

EXAMPLE 1

PROVIDING CELL LINES CO-EXPRESSING FACTOR VIII:C HEAVY CHAIN AND LIGHT CHAIN

Transfection-procedure

The DHFR$^-$ CHO cell line DG44 (G. Urlaub et al., Som Cell Mol Genet (1986) 12:555-566) was first transfected with the plasmid pCMF8-80AT: In this plasmid the CMV promoter (described in example 7 of WO91/07490) transcribes the FVIII-LC cDNA derived from pSVF8-80AT (described in example 6 of WO91/07490) and downstream is placed the Ad-MLP/dhfr casette derived from pAd-DHFR (described in example 4 of WO91/07490). The transfection method used was the polybrene method of W. CHaney et al. (Som Cell Mol Genet (1986) 12:237-244). By selection of DHFR$^+$ cells (DMEM + 10% DFCS) several FVIII-LC producers were isolated; one of these was designated 11W.

In order to introduce FVIII-HC in 11W the cell line was cotransfected with the plasmid pPR78 (this plasmid is an analog to pCMVF8-80AT, but harbours instead of the FVIII-LC cDNA the FVIII-HC cDNA derived from pCMVF8-92R described in example 8 of WO91/07490) and pSV2-neo (P.J. Southern and P. Berg, J Mol Appl Genet (1982) 1:327-341). The transfection method used was the modified calcium phosphate procedure of C. Chen and H. Okayama (Mol Cell Biol (1987) 7: 2745-2752). Transfectants were isolated in medium containing 700 µg Geneticin (G418 Sulphate, Gibco) per ml. Cells from the primary pool were subcloned by

the limited dilution method and th individual clones wer tested for expression fo active FVIII. In this way several FVIII:C producing cell lines were isolated and two of these were designated "45" and "57", respectively.

The cells selected in this way on the basis of the expression level were seeded into T-flasks or spinners for cultivation in the absence of heparin or in the presence of heparin in various concentrations.

The description of transfection referred to in WO91/07490 is hereby incorporated by reference, including the reference to the plasmide pSVF8-92, pSVF8-80 and pSVF8-200 deposited under the accession number ATCC 40222, ATCC 40223 and ATCC 40190, respectively.

EXAMPLE 2

CULTURING CELL LINES CO-EXPRESSING FACTOR VIII:C HEAVY CHAIN AND LIGHT CHAIN

The cell line designated "45" co-expressing Factor VIII:C Heavy Chain and Light Chain was cultivated in suspension at 37°C in cell factories in DMEM + 10% dialysed fetal calf serum in th conventional manner, and cells were harvested by trypsinization and resuspended in 100 ml TECHNE spinners at a density of 2 million cells per ml in DMEM (Gibco 074-90024T) supplemented with 110 mg/l Na-pyruvate, 150 mg/l l-proline, 3.7 g/l $NaCH0_3$, 1.4 g/l tryptose phosphate, 5 mg/l insulin, 0.5 g/l 6-amino-hexanoic acid and 2% NBS heat inactivated at 56°C for 30 minutes. The cultures were incubated at 27°C. Samples were taken over a p riod of 9 days. Various amounts of heparin were added.

The results of the titration of the heparin effect is shown in Figure 1. It is seen that the optimal concentration of heparin

in this type of culture is 1-2 IU/ml where an increase of the level of expressed Factor VIII:C units by 50% is seen. The effect of heparin is clearly less pronounced in a concentration of 5 and 10 IU/ml.

EXAMPLE 3

The isolated cell line designated "57" co-expressing Fact r VIII:C Heavy Chain and Light Chain was cultivated in t-flasks in DMEM + 10% dialysed fetal calf serum in the conventional manner to confluence. Confluent t-flasks were shifted to the same medium as in Example 2, and incubated at the same temperature in the presence and absence of heparin. The cultures w r fed fresh medium with 3 days intervals. Samples were taken for FVIII assays prior to media change.

Table 1 shows the increased FVIII:C levels obtained from t-flask cultures when heparin is added in low concentration (2 IU/ml). The increase is to a level of 113 to 138% of the FVIII level of the control without heparin addition as a function of the cultivation time in the presence of heparin.

Table 1

Increased yield of Factor VIII:C in T-flask culture in the presence of 2 IU/ml heparin

Harvest on day	FVIII:C U/ml + hep	FVIII:C U/ml - hep	Yield on hep. add. in % of yield of control

3	9.3	8.2	113
7	22.3	17.5	128
10	23.5	17.1	138

EXAMPLE 4

The isolated cell lines designated "45" and "57" co-expressing Factor VIII:C Heavy Chain and Light Chain were cultivated in suspension as explained above in the presence and absence of heparin.

As compared to Example 3, the increase in FVIII level is even more pronounced when the cells are cultured in suspension. As seen from tables 2 and 3, the increase in FVIII level caused by addition of heparin is 120 to 193% of the control, where no heparin is added.

Tables 2 and 3 also indicate that the increase of the Factor VIII:C level is in excess of the increased expression of the Factor VIII:LC and Factor VIII:HC when co-expression in the presence of heparin.

Table 2

Increased yield of Factor VIII:C and of Factor VIII:LC and Factor VIII:HC when cultivating cell line "45" in suspension culture in the presence of 2 IU/ml heparin.

Time days	FVIII:C yield in % of yield of control	FVIII:LC yield in % of yield of control	FVIII:HC yield in % of yield of control
0			
4	120	97	129
5	137	101	128
7	157	108	86
10	193	158	132

Table 3

Increased yield of Factor VIII:C and of Factor VIII:LC and Factor VIII:HC when cultivating cell line "57" in suspension in the presence of 2 IU/ml heparin

Sample on day	FVIII:C yield in % of yield of control	FVIII:LC yield in % of yield of control	FVIII:HC yield in % of yield of control
3	118	106	128
4	127	126	128
5	148	134	86
6	162	119	111

EXAMPLE 5

STABILIZING EFFECT OF HEPARIN ON FACTOR VIII:C HEAVY CHAIN DURING COEXPRESSION OF FACTOR VIII:C HEAVY CHAIN AND FACTOR VIII:C LIGHT CHAIN

3.5 cm's dishes were seeded with cell line "45" to confluency in the following media: DMEM + 2% NCS + 5 mg/l insulin + 1.4 g/l tryptose phosphate (TP) and in the same medium also add d 2 U/ml heparin. After incubation over night at 37°C, the dishes were incubated at 27°C for five days for adaptation. After two washes each dish was labeled with 80 μCi ^{35}S-methionine for 20 hours in the following methionine-free media:

1. DMEM+2% NCS+5mg/l insulin+1.4 g/l TP
2. DMEM+2% NCS+5 mg/l insulin+1.4 g/l TP+2 IU/ml heparin

After the labelling period the media were collected for immuno-precipitation with a dog polyclonal antiserum to human FVIII; this antiserum binds both the complex of the subunits and free heavy and light chains of FVIII. The precipitated samples were loaded on a 10% SDS gel. The resulting exposure is shown in Fig. 2. The 92 kD HC and the 80 kD LC doublet are seen in all lanes. Comparing lanes 1 and 2 it is seen that the amounts of the 50/43 kD bands originating from the HC are much more pronounced in lane 1 than in lane 2, indicating that heparin has suppressed the activating cleavage of the heavy chain and hence, a greater fraction of the subunit-complex is found as the more stable complex of the 92 kD and 80 kD subunits.

EXAMPLE 6

Increased Yield of Factor VIII:C in T-flash Culture i Serum Free Medium Containing Heparin and Lipoprotein.

PREPARATION OF EGG YOLK LIPOPROTEIN FRACTION

100 ml egg yolk and 200 ml PBS were stirred for 30 min. PEG 6000 was added to 3.5%, stirred for 60 min. and centrifuged at 3000 RPM for 30 min.

The sediment was dissolved in 200 ml PBS, stirred for 30 min. and spun down at 10,000 RPM for 20 min.

The sediment was redissolved in 200 ml 1 M NaCl and stirred overnight at 40° C. The mixture was then centrifuged at 10,000 RPM for 20 min. and the supernatant was sterilised by filtering through 0.2 μ pore size filter.

This lipoprotein fraction was used in the below culturing.

The cell line designated "57" co-expressing Factor VIII:C Heavy Chain and Factor VIII:C Light Chain was cultured in T-80 flasks in serum containing medium. After reaching confluency the cells were adapted to production conditions for three days. Production medium and conditions were described in Example 3, except that there was no addition of serum and the basal medium was DMEM/F-12. 5% addition of the egg yolk fraction containing lipoprotein was tested alone and together with 1, 2 and 5 IU heparin, and for comparison the same medium without lipoprotein was tested using 1, 2 and 5 IU heparin. Serum free medium without lipoprotein and heparin was used as control. The medium was changed and samples were taken on day 2 and day 4 and assayed for FVIII:C (coa) activity.

In samples containing lipoprotein (egg yolk fraction) the FVIII:C activity increased 60-70% when adding 1 IU/ml h parin, 80-100% when adding 2 IU/ml and 6-32% when adding 5 IU/ml. The addition of heparin alone did not incr ase the FVIII:C activity. Thus, the combined effect of addition of lipoprotein and heparin is a more than additive effect of the two separate components.

Sample No.	Medium Additions	FVIII:C U/ml Day 2	FVIII:C U/ml Day 4
1	5% LP	4.21	6.14
2	5% LP + 1 IU/ml heparin	7.17	9.70
3	5% LP + 2 IU/ml heparin	8.43	11.10
4	5% LP + 5 IU/ml heparin	4.47	8.12
5	None	0.39	1.43
6	1 IU/ml heparin	1.20	1.32
7	2 IU/ml heparin	0.78	1.64
8	5 IU/ml heparin	0.99	2.02

LP = Lipoprotein, egg yolk fraction.

EXAMPLE 7

Increased Yield of Factor VIII:C in T-flash Culture i Serum Free Medium Containing Heparin and an egg yolk fraction free of lipoproteins.

PREPARATION OF THE LIPOPROTEIN FREE EGG YOLK PROTEIN FRACTION

100 ml egg yolk and 200 ml PBS were stirred for 30 min. PEG 6000 was added to 3.5%, stirred for 60 min. and centrifuged at 3000 RPM for 30 min.

The supernatant is the lipoproteinfree egg yolk protein fraction (SUP 0).

This proteinfraction was used in the below culturing.
The cell line designated "57" co-expressing Factor VIII:C Heavy Chain and Factor VIII:C Light Chain was cultured in T-80 flasks in serum containing medium. After reaching confluency the cells were adapted to production conditions for three days. Production medium and conditions were described in Example 3, except that there was no addition of serum and the basal medium was DMEM/F-12. A 5% addition of the egg yolkprotein fraction was tested alone and together with 1, 5 and 10 IU heparin. The medium was changed and samples were taken on day 2 and day 4 and assayed for FVIII:C (coa) activity.

In samples containing SUP 0 the FVIII:C activity increased markedly on addition of heparin giving an increased level of Factor VIII:C activity. This effect is reduced when adding as much as 10 IU heparin.

5 % sup 0	Heparin IU/ml	FVIII:C U/ml day 2	FVIII:C U/ml day 4
+	0	4.7	11.2
+	1	6.7	17.6
+	5	7.0	17.3
+	10	5.0	6.6

CLAIMS

1. A method for effecting an increased expression of recombinant proteins having Factor VIII:C activity in a host cell being able to express said protein comprising culturing said host i a cell growth medium comprising heparin in a concentration below 10 IU/ml so as to express said protein.

2. The method as claimed in claim 1 wherein the concentration of heparin is from 0.5 to 8 IU/ml.

3. The method as claimed in claim 2 wherein the concentration of heparin is from 1 to 2 IU/ml.

4. The method as claimed in any of claims 1-3 wherein the cell growth medium comprises serum.

5. The method as claimed in claim 4 wherein the cell growth medium comprises lipoprotein.

6. The method as claimed in any of claims 1-3 wherein the cell growth medium is a serum free medium supplemented with lipoprotein, von Willebrand Factor or phospholipid, or a combination of two or more of these.

7. The method as claimed in claim 6 wherein the growth medium is a serum free medium supplemented with lipoprotein.

8. The method as claimed in any of the preceding claims wherein the protein having Factor VIII:C activity produced is a complex of the 92 kD and 80/77 kD subunits of Factor VIII:C.

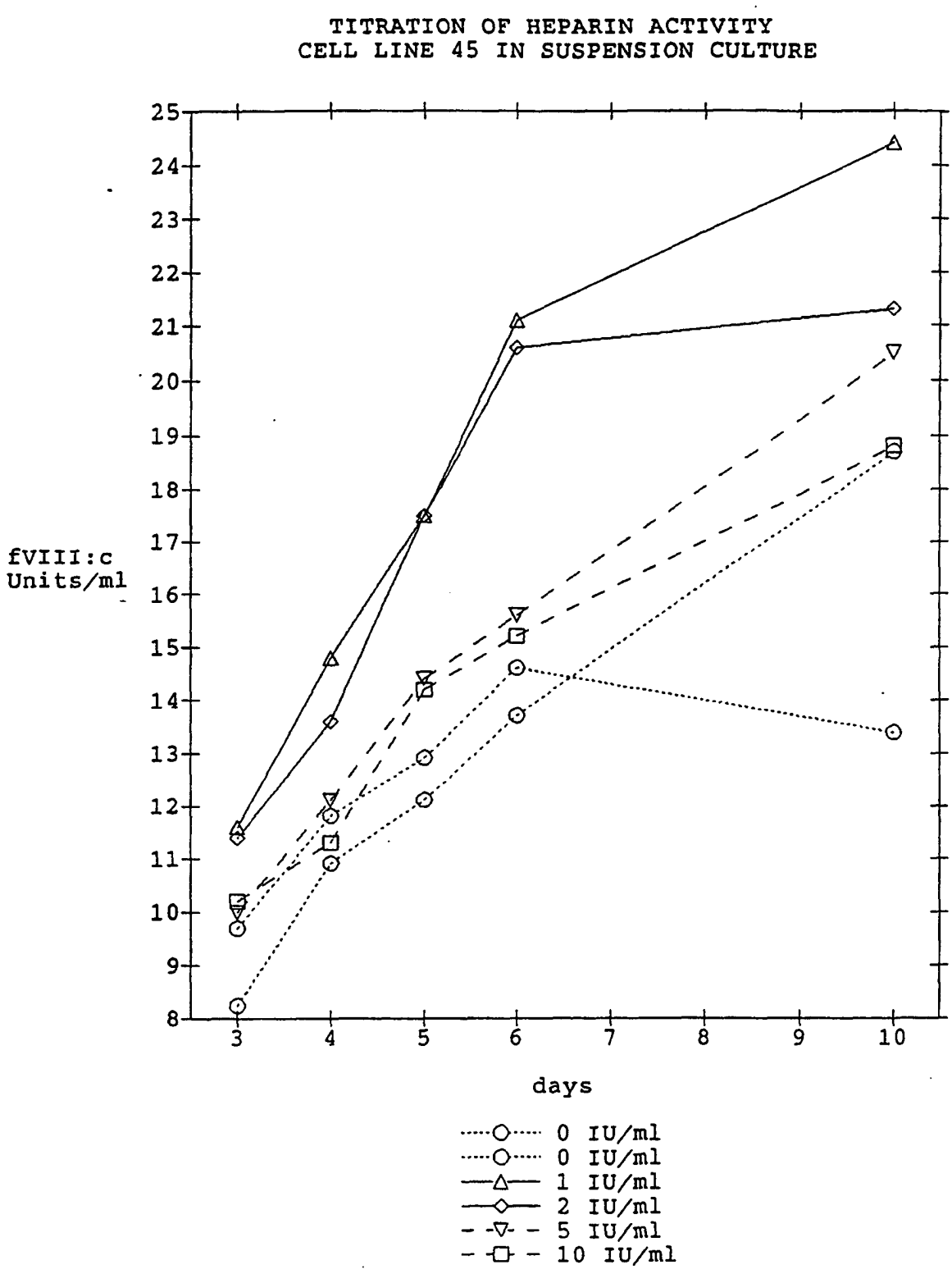
TITRATION OF HEPARIN ACTIVITY
CELL LINE 45 IN SUSPENSION CULTURE
fVIII:c
Units/ml
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
3
4
5
6
7
8
9
10
days
0 IU/ml
0 IU/ml
1 IU/ml
2 IU/ml
5 IU/ml
10 IU/ml

Fig. 1

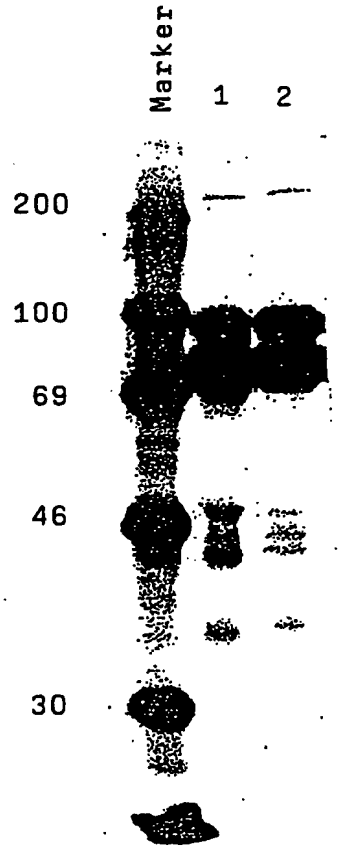

Fig. 2

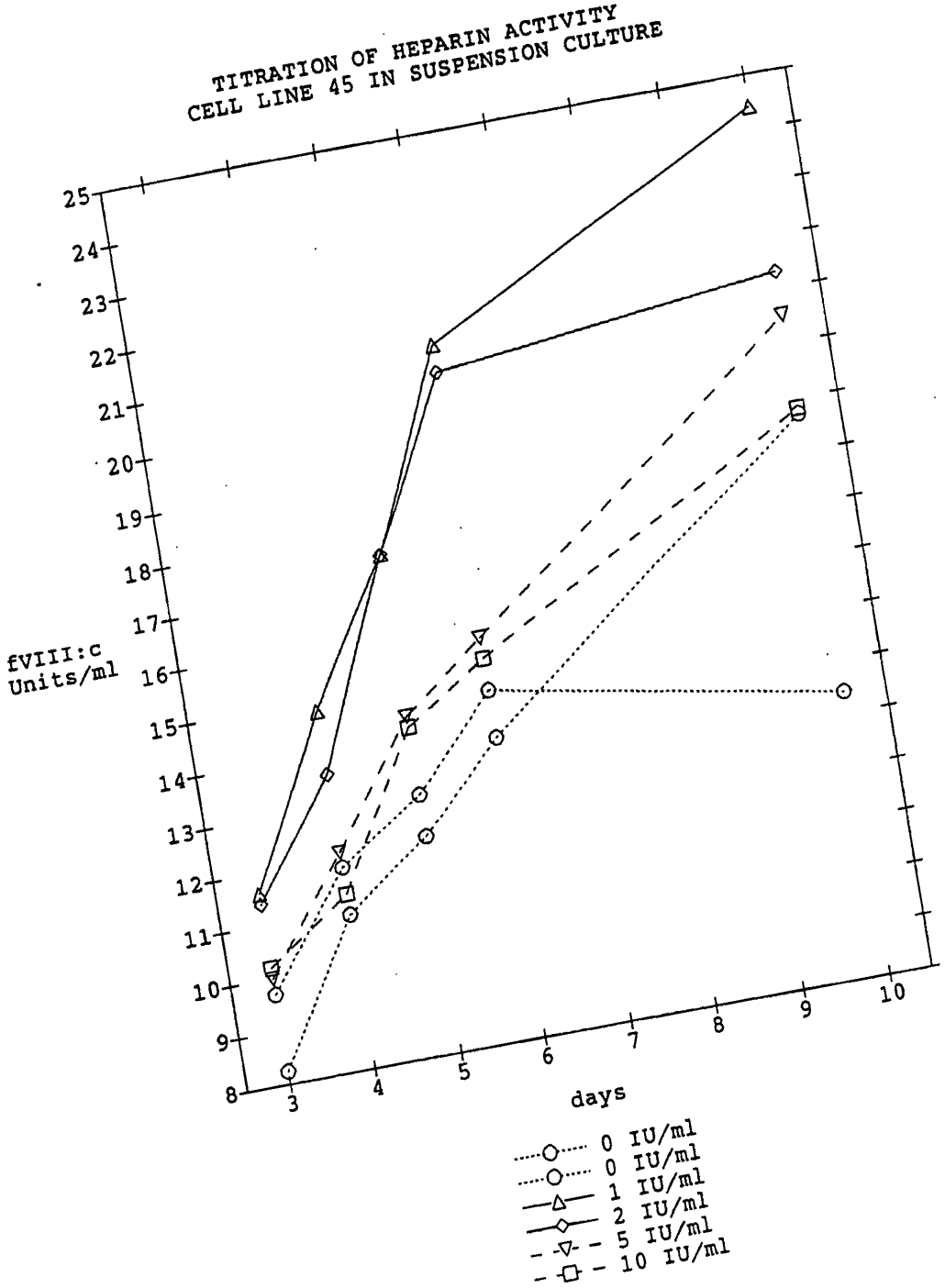
TITRATION OF HEPARIN ACTIVITY
CELL LINE 45 IN SUSPENSION CULTURE
fVIII:c
Units/ml
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
3
4
5
6
7
8
9
10
days
0 IU/ml
0 IU/ml
1 IU/ml
2 IU/ml
5 IU/ml
10 IU/ml

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00375

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A1, 0254076 (MILES LABORATORIES, INC.), 27 January 1988 (27.01.88) --	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/01/94

International application No.
PCT/DK 93/00375

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0251843	07/01/88	FR-A,B- 2599754	11/12/87
		JP-A- 63022195	29/01/88
		FR-A- 2613379	07/10/88
WO-A1- 8906547	27/07/89	AU-A- 2944789	11/08/89
		EP-A,B- 0401232	12/12/90
		SE-T3- 0401232	
EP-A1- 0441695	14/08/91	FR-A- 2657884	09/08/91
EP-A1- 0303540	15/02/89	SE-T3- 0303540	
		DE-A- 3880922	17/06/93
		FR-A,B- 2619314	17/02/89
		JP-A- 1079124	24/03/89
		US-A- 5112950	12/05/92
EP-A1- 0254076	27/01/88	SE-T3- 0254076	
		JP-A- 63042699	23/02/88